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A species-specific satellite DNA family in the genome of the coffee root-knot nematode *Meloidogyne exigua*: application to molecular diagnostics of the parasite

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SUMMARY

A new *Bgl*II satellite DNA has been isolated, cloned and sequenced from the coffee root-knot nematode, *Meloidogyne exigua* (Nematoda: Tylenchida). It is represented as tandemly repeated sequences with a monomeric unit of 277 bp. The monomers are present at approximately 17 900 copies per haploid genome, and represent about 9.7% of the total genomic DNA. Twenty randomly chosen monomers have been sequenced. The deduced unambiguous consensus sequence is 277 bp long, and displays an A + T content of 54.2%. The monomers are very homogenous in sequence, showing on average 2.4% divergence from their consensus. Therefore, it is hypothesized that this repeated family may have recently appeared in the genome of the nematode, through some extensive amplification burst. Using a cloned monomer as a probe, dot-blot experiments demonstrated the species-specific distribution of the *Bgl*II satellite DNA. Moreover, squash-blot assays allowed us to detect single *M. exigua* individuals, at any developmental stage, and even within root tissues, without the need for preliminary DNA purification. From these results, it is concluded that the procedure described, using the satellite DNA as a sensitive species-specific probe, should constitute an improved and accurate diagnosis method for the detection and identification of the nematode, which would contribute to the implementation of targeted pest management strategies in all coffee growing countries of South and Central America.

INTRODUCTION

Nematodes belonging to the genus *Meloidogyne*, commonly known as root-knot nematodes (RKN), constitute the most widely distributed and damaging group of plant-parasitic nematodes, being responsible for at least 5% of crop losses on a worldwide basis (Sasser and Carter, 1985). These sedentary biotrophic endoparasites have evolved very complex relationships with the root tissues of their hosts, in which they induce the formation of specialized feeding structures known as giant cells (Williamson and Hussey, 1996). The disease is characterized by galls or root-knots on infected plants. Symptoms include poor fruit yield, stunted growth, wilting, and increased susceptibility to other pathogens. On coffee (*Coffea arabica*), RKN are the most damaging nematode species, causing great losses to farmers and to the economy of developing countries. Among them, *M. exigua* is certainly the most widely distributed and serious species, present in nearly all the major coffee growing countries of South and Central America, either as separate species or mixed with other species (Campos *et al.*, 1990). Its economic importance is so great that coffee has been replaced by other crops in heavily infested areas, as has been well documented for Brazil (Campos *et al.*, 1990).

Considering the agricultural impact of *M. exigua*, and the diversity of nematode populations in the field, it would be highly useful to develop specific DNA probes for diagnostic purposes. However, little is known about the basic molecular biology of these organisms. Thus, characterization of the highly repetitive fraction of the genome of this nematode has been initiated. Tandemly arranged repetitive DNA sequences, referred to as satellite DNA (satDNA), are ubiquitous constituents of all eukaryotic genomes and are widely represented in animal and plant species (Beridze, 1986). They consist of very high repetitions of a basic monomeric unit 70–2000 bp long and appear to be clustered in heterochromatin centromeric and telomeric regions (Charlesworth *et al.*, 1994). Although no defined function has yet been established, satDNA has been suggested to be involved in evolutionary processes and the stability of genome structure (Csink and Henikoff, 1998). Because it diverged rapidly during evolution, and is constantly homogenized, it often gives rise to sequences that are species- or genome-specific (Bachmann *et al.*,

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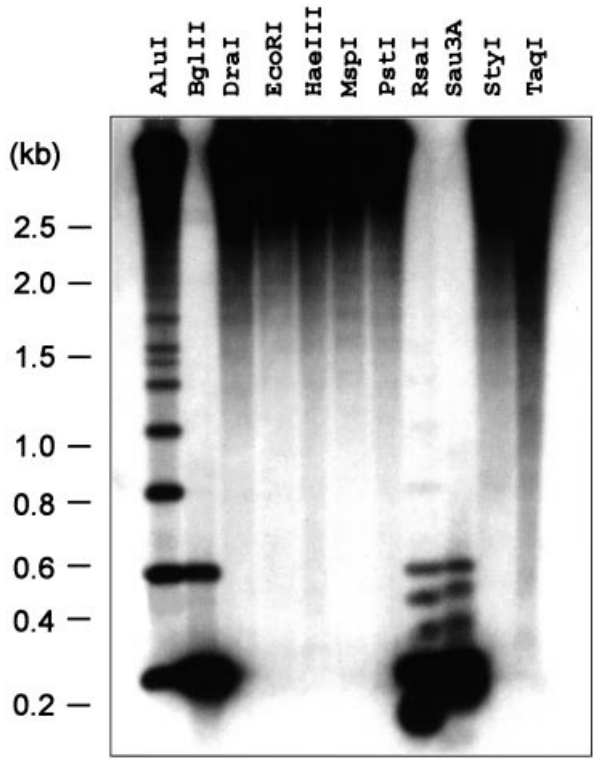

Fig. 1 Southern blot analysis of *Meloidogyne exigua* genomic DNA. For each restriction enzyme, 2 μg of total genomic DNA were digested to completion, fractionated on a 1% agarose gel, transferred on to a nylon membrane and hybridized with the ≈ 270 bp *Bgl*II fragments.

1993). In particular, such a feature may lead to the development of taxonomic markers suitable for molecular diagnostics, as has been shown in the case of nematodes of agronomic interest (reviewed in Grenier *et al.*, 1997).

In this paper, we report the identification, molecular cloning, genomic organization and sequence analysis of a new *Bgl*II satDNA from the coffee RKN *M. exigua.* We show that this sequence is species-specific and can therefore be used as an identification tool for pest management programmes. Moreover, the results of this study are compared with other RKN satDNAs in order to discuss the general evolutionary processes acting on these sequences.

RESULTS

Detection and cloning of a *Bgl*II satellite DNA in *Meloidogyne exigua*

Total genomic DNA of *M. exigua* (isolate ex1) was digested to completion with a set of 11 restriction enzymes in order to detect highly repetitive sequences. With four enzymes (*Alu*I, *Bgl*II, *Rsa*I and *Sau*3A I), the presence of a strong band of ≈ 270 bp was observed after electrophoresis (data not shown). The ≈ 270 bp *Bgl*II band, which was thought to correspond to the monomeric unit of a satellite DNA family, was purified from the gel, ^{32}P-labelled and used as a probe in the Southern blot analysis. The autoradiograph revealed the presence of a prominent ≈ 270 bp repeat in the *Alu*I, *Bgl*II, *Rsa*I and *Sau*3A I digests, that could perhaps correspond to the monomer (Fig. 1). Strong hybridization signals of a smaller size were obtained from the *Rsa*I and *Sau*3A I digests. In the *Bgl*II digest, a fragment of ≈ 550 bp was also detected, that could correspond to the dimer. In the *Alu*I digest, a ladder pattern of ≈ 270 bp putative multimers was observed (Fig. 1).

To further examine the arrangement of these sequences, DNA fragments produced after a time-course digestion of *M. exigua* DNA with *Bgl*II were hybridized with the probe described above. Autoradiography showed a typical ladder pattern, with an increasing amount of the monomer during digestion (Fig. 2), which is characteristic of repeated sequences arranged in tandem arrays. After complete digestion, monomers and dimers were present, which indicates the loss of some *Bgl*II restriction sites.

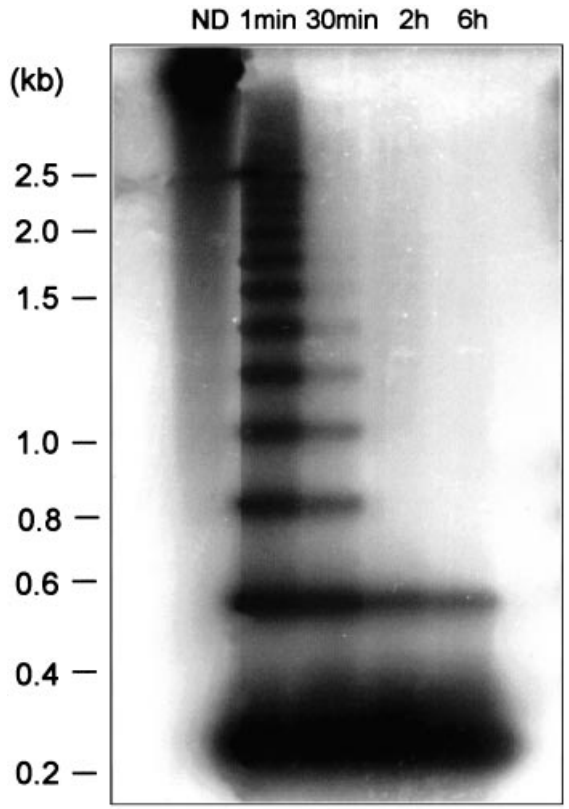

Fig. 2 Time-course digestion of *Meloidogyne exigua* genomic DNA with *Bgl*II. For each lane, 2 μg of total genomic DNA were digested, fractionated on a 1% agarose gel, transferred on to a nylon membrane and hybridized with the ≈ 270 bp *Bgl*II fragments.

Quantification and copy number in the genome

The relative abundance of the *Bgl*II repetitive sequence was assessed from dot blot experiments. Increasing amounts of a cloned monomer (pMeL8) and *M. exigua* (isolate ex1) genomic DNA were blotted on to nylon membranes and hybridized with the DNA insert released from the same clone. pBs was used as a background control and *Caenorhabditis elegans*, *Drosophila melanogaster* and calf thymus DNAs were used as negative controls (data not shown). The satellite DNA family appeared to make up to 9.7% of the nematode genomic DNA, as calculated from values obtained by scintillation measurements. Assuming that the genome size of *Meloidogyne* is about 51 Mb (Pableo and Triantaphyllou, 1989), and based on a monomer size of 277 bp (see below), this fraction corresponds to approximately 17 900 copies per haploid genome.

Monomer sequence analysis

The ≈ 270 bp *Bgl*II restriction fragments were isolated from the gel and subcloned into the pBS vector. Twenty positive clones, named pMeL(n), were selected at random and sequenced (Fig. 3). Most of these sequences were 277 bp long, except for five clones which exhibited lengths of 273 bp (pMeL13 and 15), 280 bp (pMeL3), and 281 bp (pMeL9 and 18), respectively. This length closely agreed with the estimate based on the electrophoretic mobility of the restriction fragments. An unambiguous 277 bp long consensus sequence was derived from the complete data set, and is listed at the top of Fig. 3. It was deposited in the GenBank Nucleotide Sequence Database under accession no. AY078994. The consensus has an A + T content of 54.2%.

Analysis of the primary structure of the 20 monomers in relation to the consensus sequence revealed a high homogeneity of this reiterated DNA family, with an overall average pair-wise sequence variability of only 2.4%. Compared to the consensus sequence, the individual divergence of the cloned monomers to the consensus ranged from 0.7% to 5.4%, with a total of 32 variable positions (11.6%). The variable positions were not distributed at random, but were exclusively concentrated between positions 49 and 144, and 217 and 242. Most of the mutations to the consensus sequence were single-point substitutions, except for three short deletions (1 or 4 bp) in clones pMeL3, 13 and 15, and three short insertions (4 bp) in clones pMeL3, 9 and 18. Interestingly, the same 4 bp motif (TCTT) was involved in both the insertions and deletions. In general, the observed nucleotide substitutions appeared to be shared between two or more monomers (e.g. a C-to-T substitution in positions 52 for 9 clones, or an A-to-G substitution in position 123 for 7 clones; see Fig. 3). In addition, since dimers were still present after 6 h of digestion with *Bgl*II (Fig. 2), mutations should also have occurred at the enzyme restriction site.

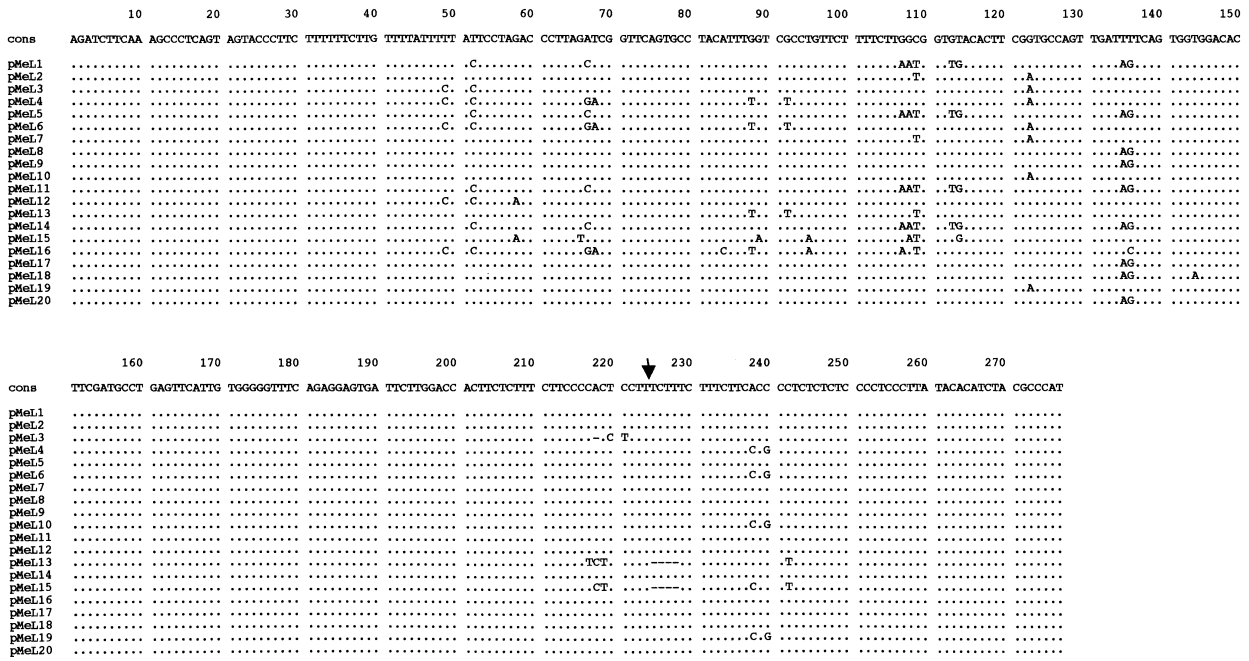

Fig. 3 Nucleotide sequences of 20 randomly cloned *Meloidogyne exigua* satellite DNA monomers, and the derived consensus sequence. In the monomer sequences, positions showing no variation from the consensus are shown with a dot and deletions are indicated with a dash. The black arrow indicates the position of a 4 bp insertion (TCTT) in clones pMeL3, 9 and 18.

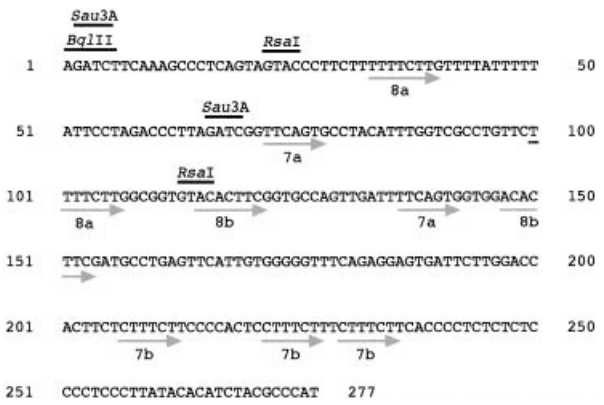

Fig. 4 Nucleotide consensus sequence of the *Bgl*II monomer of the pMeL satellite DNA family from *Meloidogyne exigua*. The 7 bp (7a, 7b) and 8 bp (8a, 8b) direct repeats are indicated with grey arrows. Restriction sites are shown with black dashes.

A computer search for evidence of internal repeated subunits in the pMeL consensus sequence allowed the detection of several direct subrepeats: two 8 bp motives repeated two times each, and two 7 bp motives repeated two and three times, respectively (Fig. 4). Analysis of the restriction sites in the consensus sequence showed a good correlation with the patterns observed on the Southern blot (Fig. 1): one *Bgl*II, two *Rsa*I and two *Sau*3A I restriction sites, respectively. No *Alu*I restriction site was detected in the consensus sequence, but four monomers (pMeL1, 5, 11 and 14) exhibited such a restriction site in positions 65–68, due to a C-to-A substitution in position 66 (Fig. 3).

A search in the EMBL and GenBank nucleic acid databases revealed no significant similarity with any recorded sequence, thus suggesting that the pMeL family represents a novel satellite DNA family in RKN.

Specific distribution of the satellite DNA and detection of single nematodes

To test the specificity of the distribution of the *Bgl*II satellite DNA in RKN, the genomic DNA of 12 isolates belonging to eight *Meloidogyne* species was dotted on to a nylon membrane and hybridized with the probe consisting of a cloned monomer (pMeL8). The autoradiography shown in Fig. 5 indicates that hybridization only occurred with the three *M. exigua* isolates tested, thus demonstrating the species-specific distribution of the pMeL satellite DNA family.

The pMeL8 probe was also tested directly on the biological material itself, using a squash-blot procedure that avoided any time-consuming DNA extraction step. As shown in Fig. 6A, either one second-stage juvenile, one female, one egg-mass or one female in galled root tissues could be unambiguously identified after 3–5 h exposure of the membrane. At the same time, the absence of cross-hybridization of the probe with non-infested roots was checked (data not shown). Moreover, the same specificity of hybridization as displayed in the previous dot-blot experiment was observed, with strong signals detected with *M. exigua* isolates only (Fig. 6A,B). In conclusion, the results of the squash-blot experiments confirmed the species-specific distribution of the *Bgl*II satellite DNA, and demonstrated the extreme sensitivity of the detection where it was used as a probe.

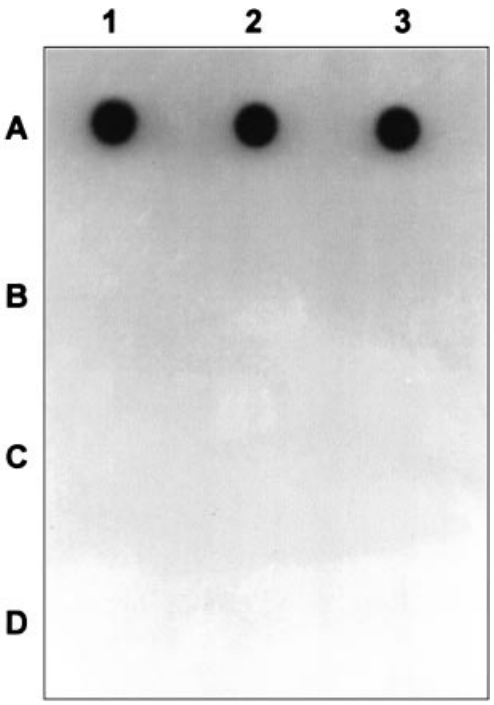

Fig. 5 Dot-blot experiment using a cloned satellite DNA monomer as a probe. Dotted genomic DNAs are as follows: A1, A2, A3 = *M. exigua* ex1, ex2, ex3, respectively; B1 = *M. arenaria* are1; B2 = *M. hapla* ha1; B3, C1, C2 = *M. incognita* inc2, inc3, inc5, respectively; C3 = *M. javanica* jav1; D1 = *M. paranaenesis* par5; D2 = *M. chitwoodi* chi1; D3 = *M. fallax* fa1. Population codes are given in Table 1.

DISCUSSION

In the present study, we have isolated and characterized a new *Bgl*II satDNA family from the coffee RKN *M. exigua*, which consists of tandemly arranged monomeric units of 277 bp. There are approximately 17 900 copies of this element, which comprise about 9.7% of the nematode genome. This value is in good agreement with the genomic content of satDNA sequences previously found in other *Meloidogyne* species, which ranges from 2.5% in *M. incognita* (Piotte *et al.*, 1994) up to 20% in *M. fallax* (Castagnone-Sereno *et al.*, 1998).

Twenty independent monomers of the *Bgl*II satDNA family were randomly selected, sequenced and compared, assuming

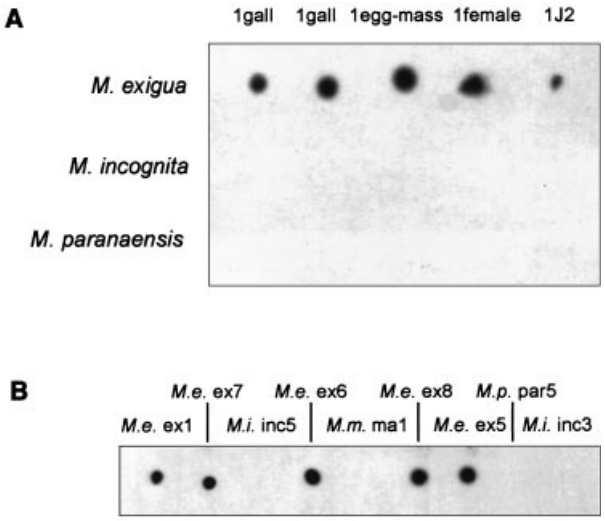

Fig. 6 Squash-blot experiments. (A) *Meloidogyne exigua*, *M. incognita* and *M. paranaensis* materials were squashed on a nylon membrane and hybridized with a cloned satellite DNA monomer. (B) Single females of *Meloidogyne exigua* (*M.e*), *M. incognita* (*M.i*), *M. mayaguensis* (*M.m*) and *M. paranaensis* (*M.p*) were squashed on a nylon membrane and hybridized with a cloned satellite DNA monomer. Population codes are given in Table 1.

that the range of nucleotidic variability they displayed was representative of the whole *M. exigua* pMeL family. The most striking result was the high degree of sequence homogeneity among all the analysed repetition units, which deviated on average from the consensus by only 2.4%. Although some sequence variability exits, no discriminating feature of any subfamily could be detected. The second remarkable trait of these sequences is their relatively low A + T content (54.2%) compared to what has been described for other RKN species. For example, the A + T content in satDNAs was 68% in *M. hapla* (Piotte *et al.*, 1994), 72.3% in *M. fallax* (Castagnone-Sereno *et al.*, 1998), and 77% in *M. incognita* (Piotte *et al.*, 1994). Since selective A + T enrichment has been suggested as a consequence of satDNA evolution (Rojas-Rousse *et al.*, 1993; Ugarkovic *et al.*, 1989), this result tends to indicate that the pMeL family is a 'young' satDNA. In that context, its relatively short life would not yet have allowed the accumulation of mutations in the repeats, which may account for the low level of variability observed among the cloned monomeric units. However, based on cytogenetic information, it has been assumed that meiotic parthenogenetic RKN, including *M. exigua*, are close relatives of the ancestor of the genus (Triantaphyllou, 1985). Therefore, it can be hypothesized that the pMeL repeats belong to a family that may have recently appeared in the genome of the nematode, through some extensive amplification burst. Indeed, the presence of internal repetitive motives in the sequence of the monomeric unit is a good indication that amplification events occurred during the evolution of this satDNA. An alternative scenario to explain such a low level of sequence divergence among the pMeL monomers is that they could have been subjected to some highly effective homogenization mechanism, such as gene conversion or unequal crossing-over, as has been proposed for a number of homogeneous satDNA families (King and Cummings, 1997; Lopez-Leon *et al.*, 1995). The observation that nucleotide changes are shared among several repeats of the pMeL family supports this hypothesis, since identical mutations shared among monomers can usually be explained as the result of partial gene conversion in the repetitive family, in contrast to independent mutational events (Drouin and Dover, 1990). However, it should be noted that homogenization mechanisms are thought to be slow for satDNA repeats (Stephan and Cho, 1994).

A survey was made of other RKN species to see whether they also contained the cloned *Bgl*II repeat. In dot-blot experiments, hybridization was detected with *M. exigua* isolates only, which strongly suggests that the satDNA sequence is specific for this taxon. However, it would be useful to test the probe against a wider range of RKN species, including other (minor) species found to be associated with coffee (e.g. *M. arabicida*, *M. konaenesis*, …), in order to definitely demonstrate its specificity. In addition, the cloned satDNA was shown to be highly abundant in the nematode genome, which allowed the unambiguous detection of single parasites independently of their developmental stage, and even in root tissues. The repeated sequence described here therefore possesses features that make it an excellent candidate for use as a specific and extremely sensitive probe for the detection and identification of *M. exigua.* Currently, the routine identification of RKN associated with coffee is performed by the microscope examination of perineal patterns of females and/or by differential host tests. However, due to the limitations inherent in these methods, *Meloidogyne* spp. populations have frequently been misidentified (Campos *et al.*, 1990). The isoenzyme electrophoresis of nematode females provides an interesting alternative analysis (Carneiro *et al.*, 2000; Esbenshade and Triantaphyllou, 1990), but this method only works with a specific stage of the nematode, and its detection threshold is not at all compatible with the identification of single individuals (Carneiro and Randig, unpublished data). Therefore, the squash-blot assay described here, using the *Bgl*II satDNA as a probe, should constitute an improved and accurate diagnosis method for the detection and identification of *M. exigua.* Since the experimental procedure is very easy and not time consuming, it should be possible to introduce it into field-work without the need for a well-equipped molecular laboratory, a step which would contribute to the implementation of targeted pest management strategies in all coffee-growing countries of South and Central America.

Table 1 Root-knot nematode (*Meloidogyne* spp.) isolates used in this study

Species	Isolate	Geographic origin
M. arenaria	are1	Pato Branco, RS, Brazil
M. chitwoodi	chi1	Valks, the Netherlands
M. exigua	ex1	Lavras, MG, Brazil
	ex2	Rondonopolis, MS, Brazil
	ex3	Lavras, MG, Brazil
	ex5	Carazo, Nicaragua
	ex6	Cajon, Costa Rica
	ex7	Cartago, Costa Rica
	ex8	Cruz Grande, Honduras
M. fallax	fa1	Baexem, the Netherlands
M. hapla	ha1	Caxias, RS, Brazil
M. incognita	inc2	Londrina, PR, Brazil
	inc3	Londrina, PR, Brazil
	inc5	Tierras Morenas, Costa Rica
M. javanica	jav1	Pelotas, RS, Brazil
M. mayaguensis	ma1	Hojancha, Costa Rica
M. paranaenesis	par5	Apucarana, PR, Brazil

EXPERIMENTAL PROCEDURES

Nematode isolates

Seventeen RKN isolates belonging to nine different species (Table 1), were maintained on tomatoes (*Lycopersicon esculentum* L. cv. Saint Pierre) grown at 20 °C in a greenhouse. They were specifically identified morphologically and according to their isoesterase electrophoretic pattern (Carneiro *et al.*, 2000). Eggs were collected from infested roots, concentrated by centrifugation at 2000 ***g*** for 2 min in a 30% sucrose solution, washed in distilled water, pelleted in a microcentrifuge and stored at −80 °C until use.

DNA purification and analysis

For each nematode isolate, total genomic DNA was purified from 100 to 200 μL of eggs, as described previously (Piotte *et al.*, 1994). Standard procedures were used for restriction endonuclease digestion, electrophoresis, transfer to nylon membranes, radioactive labelling and hybridization (Sambrook *et al.*, 1989). Hybridizations were performed overnight at 65 °C. Conditions for washing consisted of 65 °C in a 1 × SSC, 0.1% SDS final solution.

Cloning and sequence analysis

Genomic DNA of *M. exigua* isolate ex1 was digested with a set of restriction endonucleases (listed in Fig. 1), separated on a 1% agarose gel, and stained with ethidium bromide. To ensure complete digestion, incubations were performed for 4 h at 37 °C with 10 units of enzyme/μg of DNA. The most prominent band in *Bgl*II products (≈ 270 bp) was electroeluted, ligated into the plasmid vector pBS and used to transform competent *Escherichia coli* DH12S cells according to standard procedures (Sambrook *et al.*, 1989). The transformants were selected on ampicillin (100 μg/mL) agar plates containing X-gal (80 μg/mL) and IPTG (120 μg/mL), and screened by colony hybridization using the ≈ 270 bp DNA fragments isolated from the gel as a probe.

The nucleotide sequence of inserts from 20 recombinant clones selected at random and corresponding to putative monomers was determined by Eurogentec (Herstal, Belgium). Searches for restriction sites and direct repeats were performed using MacMolly, version 1.0. Multiple sequence alignments were done using Clustal W (Thompson *et al.*, 1994). DNA sequence data were compared with the EMBL and GenBank databases by using the National Center for Biotechnology Information Blast version 2.0 server (Altschul *et al.*, 1997).

Squash-blot procedure

For each nematode isolate tested, second-stage juveniles, females, egg-masses and galls were hand-picked from infested tomato roots and placed on a nylon membrane. The nematodes or the plant fragments were then ruptured by gentle pressure with a yellow, flat-tipped micropipet tip. Squashed materials were lysed by layering, successively, the filter on Whatman 3MM paper soaked with 10% SDS (2 min), 0.5 M NaOH/2.5 M NaCl (two times, 5 min each), and 3 M sodium acetate, pH 5 (three times, 2 min each). The filter was dried at room temperature (30 min) and then baked at 80 °C for 1 h. Prehybridization and hybridization of the filter were carried out as described above with a probe consisting of a ^{32}P-labelled cloned satellite DNA monomer.

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